Chapter 2

The Potential Role of Binding Proteins in Human Parasitic Infections: An In-Depth Look at the Novel Family of Nematode-Specific Fatty Acid and Retinol Binding Proteins

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Additional information is available at the end of the chapter

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1. Introduction

Nematodes (phylum Nematoda) diverged from other animals between 600–1,200 million years ago [1] and have become one of the most diverse animal phyla on earth, second only to arthropods. Many nematodes are parasites of plants, animals, and humans, posing major global ecological and economic challenges. Close to 30,000 species have been identified [1], with an estimated one million species thought to exist [2]. An astounding 16,000+ species are parasites of plants and animals, causing diseases worldwide of major socio-economic importance. In agriculture, the current financial losses caused by parasites to domesticated animals and crops greatly affect farm profitability and exacerbate challenges to global food production and distribution. Root-knot nematodes alone infect more than 3,000 plant species and cause an estimated $100 billion annual loss worldwide [3].

Nematode genomes encode for proteins specific to the phylum Nematoda, and are critically important for understanding nematode biology [4-6]. Proteins specific to the phylum Nematoda are ideal targets for drugs with low toxicity to the host and the environment. Despite the importance of nematode-specific proteins and protein families, few have been identified and very little information regarding them is available on public databases [7].

Recent studies have investigated a novel, nematode-specific family of proteins, the fatty acid and retinol binding (FAR) proteins of the phylum Nematoda. The limited information available regarding this family of proteins, as well as the areas that are poorly understood concerning their *in vivo* function(s), will be described herein. It is likely that advances in the study of this family of proteins may be significant for a broad range of scientists in understanding animal parasitism, nematode evolution and parasite-control methods.

The nematode model organism, *Heligmosomoides polygyrus*, has long been used to study nematode biology, especially as it pertains to chronic nematode infections [8,9]. It is widely believed by parasitologists that the chronicity of nematode parasite infections is directly linked to the parasite's ability to alter its immediate environment, decreasing the effectiveness of the host's responses to the invading organism. There is evidence to suggest that alteration of the parasite's environment is achieved by the release of an excretory/secretory (E/S) molecule from the parasitic nematode [8]. There is also research to support this hypothesis using the model organism *H. polygyrus*, including studies investigating the role of an E/S FAR protein from *H. polygyrus*, termed Hp-FAR-1 [10]. *H. polygyrus* is now also used as a model for studying the role of FAR proteins in nematode biology and development, nutrient acquisition and lipid sequestration [10].

It was recently discovered that parasitic nematodes of plants, animals and humans secrete these structurally novel FAR proteins into the tissues they occupy [11].This family of proteins has been shown to be highly conserved across animal parasitic nematodes. The lipid-binding characteristics of FAR proteins across multiple families of parasitic nematodes, and their presence at the host-parasite interface, lends support to the idea that this nematode-restricted family of proteins may play a crucial role in the life cycle of the roundworms, providing essential lipids they cannot synthesize themselves [12], and possibly directly in the parasitism of their animal hosts. It is speculated by researchers that these proteins may interfere with intercellular lipid signaling to manipulate the defense reactions of the host or acquire essential lipids for the nematodes [10,13,14].

The highly conserved nematode-specific FAR proteins are thought to play a critical role in lipid sequestration and delivery from the host to the nematode of a broad range of essential fatty acids, retinoids and other nutrients. Hydrophobic lipophilic molecules such as fatty acids, eicosanoids, retinoids and steroids have important functions both as energy sources and in metabolic signaling [15]. They affect fundamental processes such as gene transcription, cell development, inflammation and the immune response [16-18].

2. Previous research on parasitic FAR proteins

To support the growing evidence that FAR proteins play an active role in nematode development and survival when in contact with host tissue, researchers have performed RT-PCR to determine the developmental stage specificity of *hp-far-1* mRNA transcription in third stage larvae (L_3), fourth stage larvae (L_4) and adult *H. polygyrus* [10].These data obtained provide further evidence to suggest that FAR proteins may play a crucial role in nematode lifecycle. This research demonstrates the transcription of the *hp-far-1* gene from the L_3 larval stage of *H. polygyrus*, as well as the L_4 and adult stages of the parasite. In addition, these studies analyzed the production of a FAR protein during stages in which the parasite is embedded within the host tissue, potentially in direct contact with mediators of host immunity [10]. These findings provide a context by which FAR proteins could theoretically sequester nutrients and fatty acids, as well as alter tissue environments, based on the sequestration or delivery of small lipid ligands, as previously speculated [11,14,19].

Based on secondary structure analysis, the structure of FAR proteins are clearly unlike that of any family of lipid-binding proteins, and also structurally distinct from proteins known from any organisms- from bacteria to plants to humans, increasing the possibility that they may present useful targets for drug development [20].

Below is an example of an abbreviated, ClustalW alignment demonstrating the conservation of amino acids from human parasitic FAR proteins across the phylum Nematode (Figure 1):

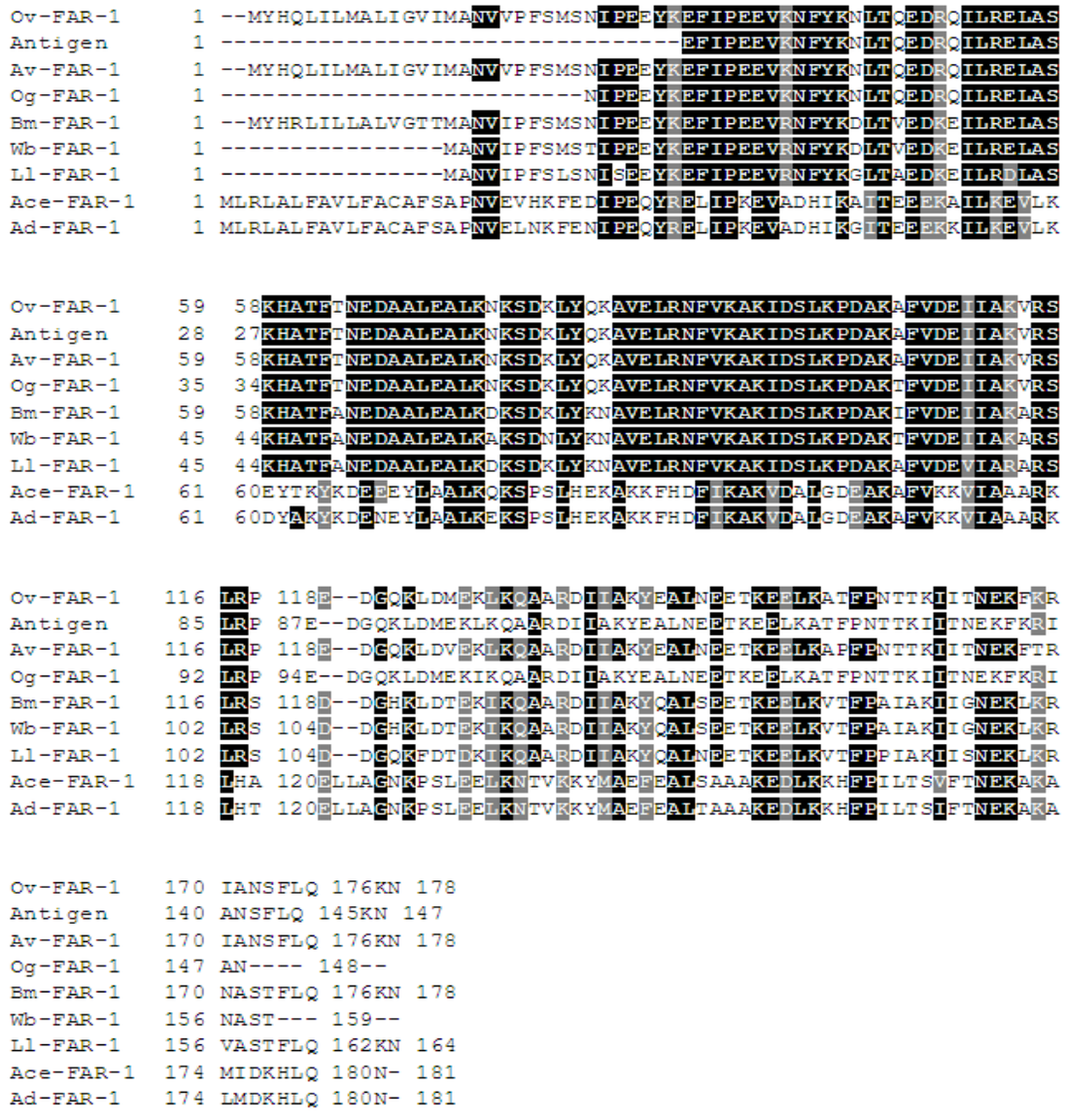

Figure 1. Amino acid sequence alignment of putative homologs of human parasitic FAR proteins from *Onchocerca volvulus* (Ov-FAR-1, AAC32662 and Antigen Maltose Binding Protein "Antigen", AAA65186), *Anthocheilenema vitae* (Av-FAR-1, AAM28244), *Onchocerca gutturosa* (Og-FAR-1, AAL33789) *Brugia malayia* (Bm-FAR-1, U69169), and *Wuchereria bancrofti* (Wb-FAR-1, AAL33794), *Loa loa* (Ll-FAR-1, AAK84218) *Ancyclostoma ceylanicum* (Ace-FAR-1, AAC76809), *Ancylostoma duodenale* (Ad-FAR-1, ABR87004). Sequences were aligned using ClustalW with a filter of 80% identity and prepared for display by BOXSHADE. Identical amino acids are shaded in black, and similar substitutions in gray. Amino acids common to every sequence are marked by an asterisk below the alignment.

There is not three-dimensional structural information available for parasitic FAR proteins and there is little information available regarding key amino acids involved in the function of parasitic FARs. The empirical structural information that exists for FAR proteins is a circular dichroism analysis showing that the Ov-FAR-1 protein from *Onchocerca volvulus*, demonstrating that it is rich in alpha-helices [21].

A study conducted in 2009 reported the X-ray crystallographic structure of Ce-FAR-7 from *C. elegans*, however, this is a non-parasitic nematode belonging to a different evolutionary group [15] Parasitic nematodes possess one or two types of FAR proteins [11,14], but the free-living *C. elegans* produces eight FAR proteins (Ce-FAR-1 to 8) [13]. They belong to three groups, group A (Ce-FAR-1, 2 and 6), group B (Ce-FAR-1, 4 and 5) and group C (Ce-FAR-7 and 8) [13]. Group A has the highest sequence identity to FARs from parasitic nematodes, such as Hp-FAR-1, Ov-FAR-1 (*O. volvulus*, human parasite) and Gp-FAR-1 (*Globodera pallida*, plant parasite) [21]. Although the sequence identity among parasitic FARs is high [14,21], the sequence identity of Ce-FAR-7 (the only FAR protein for which structural information is available) with other FAR proteins is extremely low [15], making data on Ce-FAR-7 more complicated for use in identifying key functional amino acids. In fact, of all of the *C. elegans* FAR proteins, Ce-FAR-7 is the least representative of known FAR proteins [13,14,21]. In contrast to other FAR proteins, Ce-FAR-7 exhibits minimal binding of 11-((5-dimethylaminonaphthalene-1-sulphonyl)amino)undecanoic acid (DAUDA) and, although Ce-FAR-7 induces fluorescence emission enhancements in cis-parinaric acid and retinol, it does so much less so than other FAR proteins [13]. Thus, is has been speculated that Ce-FAR-7 is functionally discrete from others [13].

In addition, a previous study investigating key amino acids in FAR ligand binding was performed on the FAR protein from the plant parasitic nematode *G. pallida*, Gp-FAR-1 [14]. In this study, several amino acids in the protein were altered by site-directed mutagenesis and, although the intrinsic fluorescence analysis of one mutant protein into which a tryptophan residue had been inserted supported computer-based predictions of the position of this residue at the protein's interior, the remaining substitutions produced no marked changes in ligand binding [14]. Therefore, this study concluded that the amino acids they had selected did not have any direct and essential involvement in the binding of ligands [14].

Thus, as it stands, there is a significant dearth of information available detailing amino acids that play a key role in ligand binding and protein function for FARs. Future work is likely to provide information on the key amino acids involved in fatty acid and lipid binding, which is essential to a basic understanding of FAR function and evolution, and the ability to utilize FAR proteins as a target for nematode-control measures.

To date, several FAR proteins, from a multitude of different nematode species, have been identified, recombinantly produced, isolated, and characterized [10,11,13,14].

FAR proteins discovered have been analyzed for fatty acid and retinol binding capabilities using spectrofluorimetry and fluorescence-based ligand-binding assays. Most commonly, fluorescence emission spectra were recorded at 20°C with the fluorescent fatty acid analog DAUDA or retinol diluted in PBS, as well as oleic acid diluted in PBS for competitive binding studies [10,11,13,14].

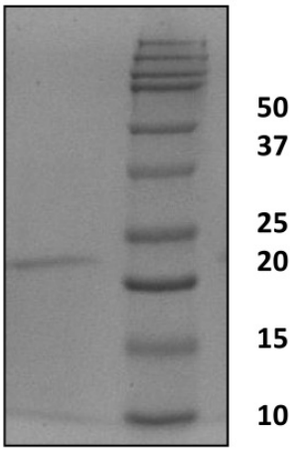

Image from Bath et al. 2009 Identification of an Immunogenic, Secreted Fatty Acid and Retinol-Binding Protein (Hp-FAR-1) from Heligmosomoides polygyrus. J. Nemat. 41(3):228-233.

Figure 2. Example of an SDS-PAGE of recombinant His-tagged FAR protein (in this case, Hp-FAR-1), produced by nickel-affinity chromatography.

Figures 3-5 demonstrate the specific results obtained for the FAR protein from *H. polygyrus*, Hp-FAR-1, which are fairly representative of binding patterns displayed by nematode FAR proteins.

Figure 3 shows Hp-FAR-1 binding characteristics, which are typical for parasitic FAR proteins, demonstrating the FAR protein bound to the fluorophore-tagged fatty acid DAUDA to produce a significant blue shift in its peak emission from 543 nm to 484 nm, which is unusually large for lipid transporter proteins but typical for FAR proteins [11,13,14,21], and indicative of a highly apolar binding site. Figure 4 shows that the addition of oleic acid to Hp-FAR-1:DAUDA complexes displaced DAUDA from the protein's binding site very efficiently, and complete replacement was observed at a ligand:competitor ratio of approximately 1:9. Figure 5 shows binding of retinol by Hp-FAR-1 which is indicated by a substantial increase in fluorescence emission intensity when retinol is added to a solution of Hp-FAR-1 in buffer [10].

Phosphorylation is one of the most common post-translational modifications that occurs on cellular proteins [22]. This process causes changes in the function, activity, localization, and/or stability in about 30 percent of cellular proteins [23]. A preliminary paper investigating the more distantly related Ce-FAR-7 protein presents evidence to suggest that retinol binding is positively regulated by CKII phosphorylation at a conserved site located near the binding pocket associated with retinol binding [15]. Previous research has also demonstrated that this is 100% conserved across all plant, animal and human parasitic nematode FAR proteins known to date [10,14,15,19].

Given this high conservation of the CKII phosphorylation site, and the overall high identity of the orthologs involved, it is reasonable to assume that CKII phosphorylation may also play a role in parasitic FAR:retinol binding. However, such studies have not yet been conducted, and basic questions regarding the role of the phosphorylation in parasitic nematodes, the impact of phosphorylation of K_ds of ligand binding, and possible shifts in preference for ligands have not been analyzed.

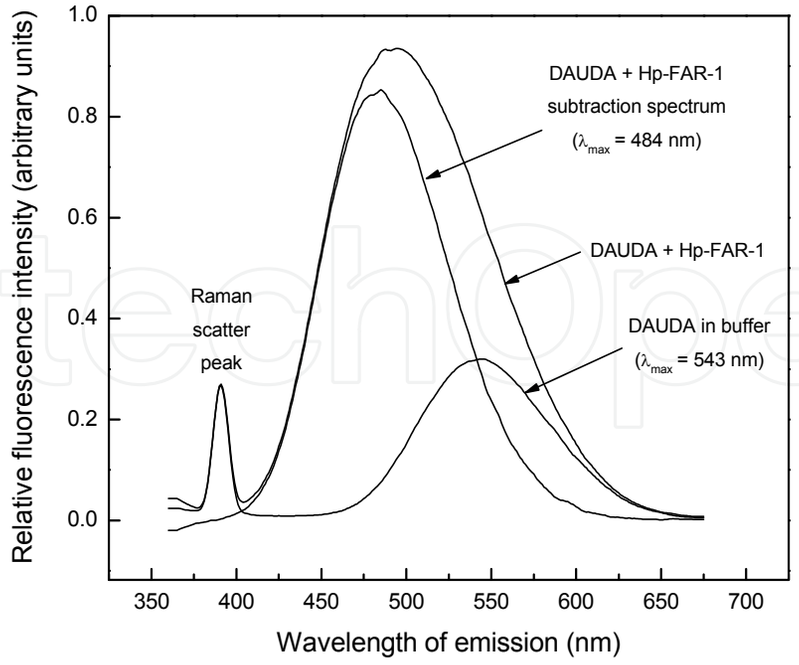

Figure from Bath et al. 2009 Identification of an Immunogenic, Secreted Fatty Acid and Retinol-Binding Protein (Hp-FAR-1) from Heligmosomoides polygyrus. J. Nemat. 41(3):228-233.

Figure 3. Binding of DAUDA by Hp-FAR-1 indicated by a substantial increase in fluorescence emission by DAUDA and the subtraction spectrum showing a significant blue shift in peak emission.

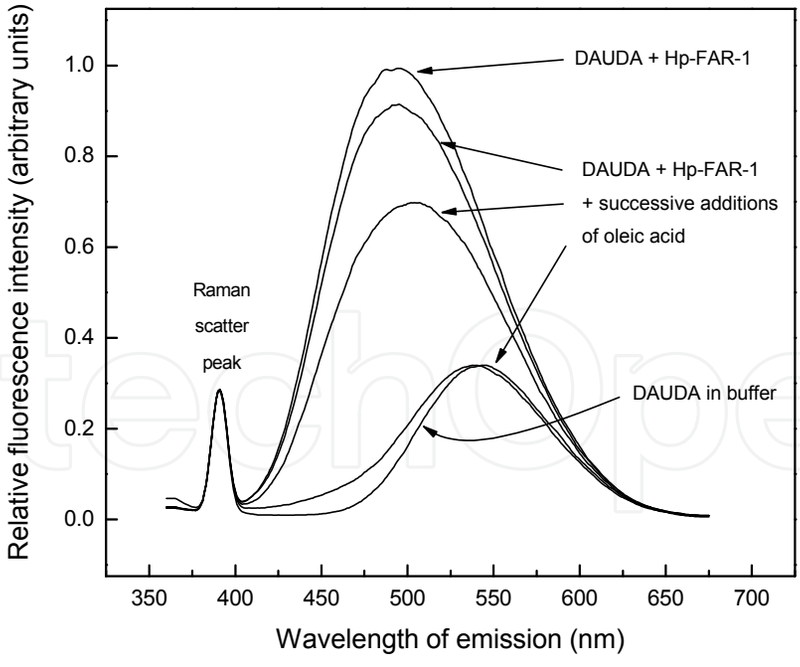

Figure from Bath et al. 2009 Identification of an Immunogenic, Secreted Fatty Acid and Retinol-Binding Protein (Hp-FAR-1) from Heligmosomoides polygyrus. J. Nemat. 41(3):228-233.

Figure 4. Highly efficient displacement of DAUDA from Hp-FAR-1 by successive additions of oleic acid. The concentration of compounds in the cuvette were approximately 1 μM DAUDA, and approximately 0.08 μM, 0.9 μM, and 9 μM oleic acid in the successive additions.

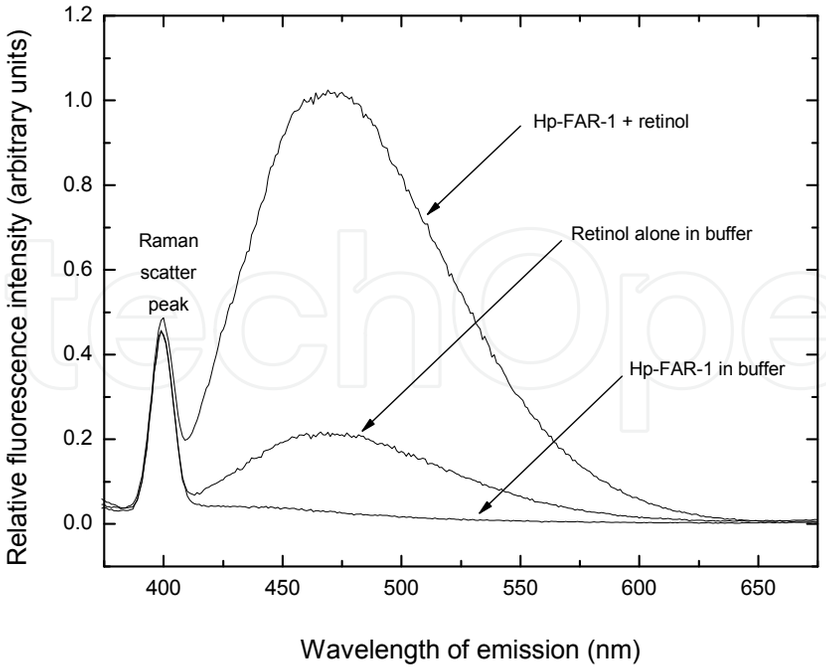

Figure from Bath et al. 2009 Identification of an Immunogenic, Secreted Fatty Acid and Retinol-Binding Protein (Hp-FAR-1) from Heligmosomoides polygyrus. J. Nemat. 41(3):228-233.

Figure 5. Binding of retinol by Hp-FAR-1 indicated by a substantial increase in fluorescence emission intensity when retinol is added to a solution of Hp-FAR-1 in buffer.

Ligand binding by Hp-FAR-1. Protein was mixed with environment-sensitive fluorescent ligands DAUDA (panel A), oleic acid (panel B) or retinol (panel C).

Analysis of the impact of phosphorylation on parasitic FAR proteins is of great interest for future studies because the initial binding capabilities of the analyzed Ce-FAR-7 are distinctly different than those of the distantly human and animal parasitic FARs, and thus one cannot make the assumption that CKII phosphorylation has the same role in parasitic FARs. While the results from Ce-FAR-7 suggest that the retinol binding site (P2) is probably regulated by CKII, the fatty acid binding pocket (P1) was not affected by phosphorylation. However, Ce-FAR-7's affinity for retinol is an order of magnitude lower than that for fatty acids, implying that, in contrast to the other FAR family members, transport of retinoids is not the major function of Ce-FAR-7 [15]. Thus, it is still unknown if the FAR protein affinity for signaling lipids, such as retinoids, is regulated in parasitic FARs, and if it is, how phosphorylation impacts the K_ds of the bound ligands.

Nematode FARs may function in scavenging, transport and metabolism of hydrophobic lipophilic molecules such as fatty acids, eicosanoids, retinoids, and steroids. These molecules have important functions as energy sources and are used in metabolic and developmental processes of embryogenesis, glycoprotein synthesis, growth and cellular differentiation [24,25]. Parasites cannot synthesize all of the lipids required for metabolism, but instead rely on a ready-made supply from their host. FAR proteins might facilitate uptake, transport and distribution of these molecules to specific target tissues [24].

Secretion of FARs into host tissues by parasites may, however, have significance beyond mere resource acquisition, in particular as depletion of these substances can significantly alter the tissue environment. For example, retinol has intrinsic importance in collagen synthesis and tissue repair mechanisms in a variety of tissues including the skin and the gut [26]. Retinol also prevents the decline in IgA and Th2 cytokine levels [27].

Cellular responses to *H. polygyrus* are an active area of research [9,28,29], and more recently there has been a surge of interest in the FAR family of proteins from organisms such as *H. polygyrus*, with respect to their potential role in altering their environment [10,11,21,30]. It is even speculated that the ability of FAR proteins to sequester RA results in cellular and cytokine alterations in the nematode's environment, a phenomenon that historically could have allowed nematodes to take advantage of a new niche, vertebrate parasitism.

3. Future directions and parasitic FARs as targets in drug development

There is an increasing interest by laboratories in investigating FAR proteins [11,14-15,30]. Continued studies will provide invaluable information as to exactly which amino acids are key in the binding of fatty acids and retinol, as well as the possible *in vivo* function(s) of nematode FAR proteins. These data would also enhance our understanding of the basic structural information for understanding the mode of action of FAR proteins and investigation of inhibitors of lipid binding.

A fuller appreciation of the function of FAR protein secretion will require a more detailed analysis and understanding of what parasitic FARs bind. Do they, for example, preferentially bind inflammatory mediators, their metabolic products or other potential pharmacologically active lipid mediators? Interference with such binding activities would likely have significant effects on the survival of nematodes and the impact of FARs on tissue dwelling and disease processes, and would further justify the relevance of targeting these molecules for drug targeting and/or vaccine development.

It is speculated, based on bioinformatic analyses, phylogenic studies, and the high conservation of FAR proteins amongst parasitic nematodes, that the evolution of FAR proteins may have been a precursor to nematode parasitism [13]. As such, it is also of interest to explore naturally occurring sequence variations, to determine which conserved regions or amino acids are imperative for ligand binding. Results from such studies are likely to shed more light on the role of FAR proteins in parasitism, with implications for their possible role(s) in the adaptation of free-living to parasitic lifestyles, by combining our knowledge of protein function with our information about sequence conservation from species to species.

Of particular interest for investigating the potential role of FARs in human parasitic diseases are the FAR affinities for the many vitamin A derivatives and fatty acids that are known to regulate expression of genes through the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). These include RA in both of its stereoisomeric forms, all-trans retinoic acid

(ATRA, binds RARs) and 13-cis-retinoic acid (binds RAR). In addition, 9-cis-retinoic acid, which is converted from 13-cis-retinoic acid (binds RARs and RXRs) [31]. Two other RAR-interacting retinoids have been identified in tissue: all-trans 13,14-dihydroretinoic acid and beta –apoands-14'-carotenal [32,33]. Phytanic acid [34], docosahexaenoic acid [35] and other unsaturated fatty acids [36] have been proposed as additional/alternative ligands for RXRs. Future research may investigate the binding abilities FARs to leukotrienes and prostaglandins, both of which are compounds involved in the inflammatory response [18].

As the screening of potential FAR ligands broadens, it will provide researchers with a much fuller appreciation of the potential function of FAR protein secretion. The information gained with respect to ligands bound would be useful in the design and analysis of FAR studies aiming to target FARs in parasites, by demonstrating the potential pathways that interference with FAR binding activities are likely to effect.

Investigating the potential role(s) of FAR proteins in parasitic nematodes at a range of physiologically relevant temperatures is an area of research that should not be overlooked, in the event that ligand binding dissociation constants (Kds) are temperature sensitive. Such an expansion of the analysis to include additional temperatures may be quite relevant, as previous research has demonstrated significant variation in the binding affinities of various proteins to their ligands with regard to environmental temperature [37], and nematode-specific FAR proteins have been shown to be heat-stable [13,20]. Given that plant-parasitic nematodes are much more prevalent during warmer seasons and in tropical climates [38-40] and that animal parasites would, by their nature, inhabit warmer environments, it seems possible that FAR studies conducted to date, at 20°C, may not accurately reflect the binding properties of this family of protein *in vivo.*

The investigation of the role of phosphorylation by comparing the Kds for the most commonly tested ligands (DAUDA, oleic acid and RA) will likely be expanded by subjecting phosphorylated FAR proteins to a broader panel of ligands, to determine if the binding preferences and Kds for a broader range of ligands are altered upon phosphorylation. This knowledge would shed light on our basic understanding of nematode development and biology, by investigating the role of the conserved CKII phosphorylation site on FAR proteins with respect to ligand binding capabilities and respective Kds. The function of the N-terminal conserved CKII phosphorylation site on FARs may be important for ligand-binding. When phosphorylated, it may increase or decrease the affinity of the binding pockets for the binding of any number of essential fatty acids, retinoids and other nutrients that nematodes are not able to synthesize on their own [12]. Such knowledge sheds light on the possible *in vivo* roles of FARs in small lipid sequestration and delivery. This information directly impacts our understanding of basic nematode biology, development, and parasitism as hydrophobic lipophilic molecules such as fatty acids, eicosanoids, retinoids and steroids have important functions both as energy sources and in metabolic signaling [15] and affect fundamental processes such as gene transcription, cell development, inflammation and the immune response [16-18].

The ability of FAR proteins to bind fatty acids and retinoids is a characteristic of particular interest to parasitologists, due to the proteins potential function in nutrient acquisition, manipulation of the parasite's environment, and countering host defense reactions [24,41,42]. Retinoids, in particular, have the potential to regulate the expression of multiple genes by various mechanisms [43], through the binding of either RARs or RXRs [28]. When bound with ligand, the receptors transactivate specific cis-elements of target genes, known as Retinoic Acid Response Elements (RAREs). It is through this mechanism that retinol acts to positively regulate the T cell-derived cytokine interleukin 4 (IL-4). This is a key finding with respect to animal and human parasitism, in that localized host IL-4 levels are directly correlated to host protection [9,28,29], and thus a parasites' ability to regulate local IL-4 levels through an E/S protein may, in fact, be key to their survival.

Continued research in this area of FAR protein function will likely provide invaluable information regarding the possible *in vivo* function(s) of nematode FAR proteins, specifically with regard to the role they play in nematode survival and their possible use as targets in parasite-control methods. Such specific information about FAR proteins and their role in nematode survival could prove instrumental for nematologists, plant and animal parasitologists, agriculturalists and disease specialists.

Our growing understanding of the nematode-specific family of protein continues to provide invaluable information regarding the possible *in vivo* function(s) of nematode FAR proteins in parasitism and disease. Enhanced understanding of FAR proteins and their role in nematode biology could prove instrumental for plant and animal parasitologists, agriculturalists, cell biologists, evolutionists, and disease specialists. Continued enlightenment will also contribute to our fundamental understanding of how the evolution of FAR proteins may have contributed to the adaptation of free living nematodes, such as *C. elegans* (having eight known FAR proteins), to parasitic lifestyles.

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